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MEETING PRESENTATION

Staphylococcus aureus Utilizes Host-Derived Lipoprotein Particles as Sources of Fatty Acids

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ABSTRACT Methicillin-resistant *Staphylococcus aureus* (MRSA) is a threat to global health. Consequently, much effort has focused on the development of new antimicrobials that target novel aspects of *S. aureus* physiology. Fatty acids are required to maintain cell viability, and bacteria synthesize fatty acids using the type II fatty acid synthesis (FASII) pathway. FASII is significantly different from human fatty acid synthesis, underscoring the therapeutic potential of inhibiting this pathway. However, many Gram-positive pathogens incorporate exogenous fatty acids, bypassing FASII inhibition and leaving the clinical potential of FASII inhibitors uncertain. Importantly, the source(s) of fatty acids available to pathogens within the host environment remains unclear. Fatty acids are transported throughout the body by lipoprotein particles in the form of triglycerides and esterified cholesterol. Thus, lipoproteins, such as low-density lipoprotein (LDL), represent a potentially rich source of exogenous fatty acids for *S. aureus* during infection. We sought to test the ability of LDLs to serve as a fatty acid source for *S. aureus* and show that cells cultured in the presence of human LDLs demonstrate increased tolerance to the FASII inhibitor triclosan. Using mass spectrometry, we observed that host-derived fatty acids present in the LDLs are incorporated into the staphylococcal membrane and that tolerance to triclosan is facilitated by the fatty acid kinase A, FakA, and Geh, a triacylglycerol lipase. Finally, we demonstrate that human LDLs support the growth of *S. aureus* fatty acid auxotrophs. Together, these results suggest that human lipoprotein particles are a viable source of exogenous fatty acids for *S. aureus* during infection.

IMPORTANCE Inhibition of bacterial fatty acid synthesis is a promising approach to combating infections caused by *S. aureus* and other human pathogens. However, *S. aureus* incorporates exogenous fatty acids into its phospholipid bilayer. Therefore, the clinical utility of targeting bacterial fatty acid synthesis is debated. Moreover, the fatty acid reservoir(s) exploited by *S. aureus* is not well understood. Human low-density lipoprotein particles represent a particularly abundant *in vivo* source of fatty acids and are present in tissues that *S. aureus* colonizes. Herein, we establish that *S. aureus* is capable of utilizing the fatty acids present in low-density lipoproteins to bypass both chemical and genetic inhibition of fatty acid synthesis. These findings imply that *S. aureus* targets LDLs as a source of fatty acids during pathogenesis.

KEYWORDS *Staphylococcus aureus*, fatty acid kinase, fatty acids, *geh*, lipase, lipoprotein particles

The Gram-positive pathogen *Staphylococcus aureus* is the leading cause of health care-associated infections and is a significant source of morbidity and mortality in the United States (1). In keeping with this, *S. aureus* infects skin and soft tissue but also has the capacity to penetrate the vasculature and colonize internal organs, including the heart, bone, and liver (2). The propensity to colonize multiple vertebrate organs is due to the ability of this pathogen to survive and proliferate in the blood. Within the

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vasculature, *S. aureus* withstands the innate immune response and hijacks host-derived metabolites needed for proliferation (3). In addition to a remarkable ability to survive in vertebrate blood, the prevalence of antibiotic-resistant *S. aureus* isolates presents additional challenges to the efficacious treatment of infection. Consequently, the development of new therapeutic strategies is needed to combat *S. aureus* infections.

Bacterial synthesis of fatty acids is a focus for the design of new therapeutics to treat infections caused by *S. aureus* and other bacterial pathogens (4, 5). Bacteria utilize the type II fatty acid synthesis (FASII) pathway for *de novo* synthesis of fatty acids and maintenance of the plasma membrane (6, 7). FASII is composed of multiple enzymes, distinguishing it from the single-enzyme type I system used by eukaryotes (8). Therefore, FASII offers several ideal candidate targets for antimicrobial development (9, 10). As such, multiple FASII inhibitors are currently being developed. Many of these molecules target FabI (11–13), an NADPH-dependent, enoyl-acyl carrier protein reductase that elongates the fatty acid chain. The effectiveness of FabI inhibition is best exemplified by triclosan, which has been broadly used in consumer and medical goods for decades (14, 15). FASII inhibitors demonstrate various degrees of efficacy in murine models of *S. aureus* infection (11, 13, 16–19). This variability is likely due to the ability of *S. aureus* to resist FASII inhibition by incorporating host-derived fatty acids (20–22). Consistent with this, FASII inhibitor bypass mutants can be isolated when *S. aureus* is cultured in a medium supplemented with exogenous fatty acids. These FASII inhibitor-resistant isolates contain mutations within FASII initiation genes, resulting in fatty acid auxotrophy (20, 22–24). Notably, mutations in FASII initiation genes resulting in fatty acid auxotrophy have also been observed in *S. aureus* strains isolated from clinical specimens (24). Together, these data demonstrate that the ability of *S. aureus* to incorporate exogenous fatty acids obscures the efficacy of FASII inhibitors (21, 22, 25, 26), underscoring the importance of increasing our understanding of the fatty acid reservoirs exploited by *S. aureus* during infection.

The incorporation of exogenous fatty acids into the cell membrane by *S. aureus* is dependent on the fatty acid kinase FakA (also called VfrB). FakA phosphorylates exogenous fatty acids, producing acyl-PO_4, which enters phospholipid synthesis (27–29). The identification of FakA substantially advanced our understanding of the mechanisms that *S. aureus* utilizes to incorporate exogenous fatty acids. However, the role of FakA in FASII inhibitor bypass has not been established. In addition, the source(s) of fatty acids accessible to *S. aureus* within the host environment is not known. Within the vasculature, free fatty acids are a minor component of the total fatty acids present (30, 31). The vast majority of fatty acids in host blood are esterified into triglycerides and cholesterol esters found within host lipoprotein particles (30–33). Low-density lipoprotein (LDL) is one type of several classes of lipoprotein particles that function as lipid transport vehicles that deliver fatty acids and cholesterol to and from host cells throughout the vasculature. The hydrophilic exterior of lipoprotein particles consists of phospholipids, cholesterol, and proteins that surround a fatty acid-rich hydrophobic core of triglycerides and cholesterol esters (33). In the host, fatty acids are released from the triglycerides and phospholipids present within lipoprotein particles by a tissue-specific family of triacylglycerol lipases (34–36). Notably, expression of secreted lipases is a clinically defining feature of *S. aureus*, and most strains encode multiple lipases (37, 38). Previous studies established that LDL particles bind to and sequester factors secreted by *S. aureus*, such as autoinducing peptides and alpha-toxin (39–43), but the capacity of LDLs to serve as an exogenous source of fatty acids for *S. aureus* has not been explored.

We hypothesized that *S. aureus* utilizes fatty acids present within lipoprotein particles. To test this hypothesis, we monitored the sensitivity of *S. aureus* cultured in the presence of human LDL to the FASII inhibitor triclosan. We show that LDLs act as a reservoir of exogenous fatty acids that increase *S. aureus* tolerance to triclosan and that this leads to incorporation of LDL-specific fatty acids in the phospholipids of *S. aureus*. Human LDLs also support the growth of fatty acid auxotrophs of *S. aureus*. Importantly, we also demonstrate that incorporation of host fatty acids from LDLs is reduced in

strains lacking the major triacylglycerol lipase, Geh. Additionally, genetic inactivation of *fakA* significantly impairs triclosan resistance in *S. aureus* cells cultured in the presence of LDLs. Thus, host-derived lipoprotein particles provide exogenous fatty acids for the maintenance of the staphylococcal membrane, increasing the tolerance of *S. aureus* to triclosan exposure. Together, these findings show that lipoprotein particles are sources of host fatty acids for *S. aureus*.

RESULTS

Lipoprotein particles protect *S. aureus* from FASII triclosan inhibition. *S. aureus* scavenges exogenous free fatty acids for incorporation into membrane phospholipids (5, 22, 44). However, little is known regarding the host-derived sources of fatty acids that are utilized by *S. aureus*. Host fatty acids are predominantly esterified into triglycerides or cholesterol, which are transported through the vasculature by lipoprotein particles, such as LDL (30–33). We reasoned that lipoprotein particles are a viable source of host-derived exogenous fatty acids for *S. aureus*. To test this, we used the FASII inhibitor triclosan to block endogenous fatty acid production (20–22, 44) and determined the capacity of LDLs to rescue staphylococcal growth. To this end, Kirby-Bauer disk diffusion assays were performed using a laboratory-derived, methicillin-resistant USA300 strain cultured on tryptic soy agar (TSA) supplemented with chicken egg yolk, a rich source of lipoprotein particles that contain esterified fatty acids (45–47). Egg yolk contains less than 0.5% free fatty acids (48). Compared to plates without supplementation, egg yolk-supplemented plates provided significant protection from triclosan (Fig. 1A). To further characterize the source of the triclosan resistance, egg yolk was separated into granule and plasma fractions. Egg yolk plasma (EYP) is enriched for low-density lipoprotein (LDL) particles, which compose ~85% of the solution. The remaining ~15% is soluble proteins (45, 47, 49–51). EYP provided protection from triclosan comparable to that provided by egg yolk (Fig. 1A). To determine the growth kinetics of the protection provided by EYP, *S. aureus* was grown in tryptic soy broth (TSB) under the following conditions: untreated, 1% EYP alone, 1 μM triclosan alone, or 1% EYP together with 1 μM triclosan. We chose to perform our growth curves with 1 μM triclosan, as this concentration is below the concentration (7 μM) that has been described to damage the *S. aureus* cytoplasmic membrane (52). Cells cultured in the presence of 1% EYP grew similarly to untreated cells, but triclosan treatment impaired growth for up to 12 h. The addition of EYP restored the growth of *S. aureus* in the presence of triclosan (Fig. 1B). These results suggest that lipoprotein particles derived from chicken egg yolk serve as a source of exogenous fatty acids for *S. aureus*.

We next sought to test the capacity of human-derived lipoprotein particles to rescue *S. aureus* from FASII inhibition. We performed a similar growth assay with commercially available, highly purified human LDL as a source of exogenous fatty acids. *S. aureus* cells cultured on TSB supplemented with 0.34 μg/μl of purified human LDLs grew slightly greater than untreated cells, indicating that human LDLs are not toxic to *S. aureus* (Fig. 1C). Notably, the addition of human LDLs restored the growth of triclosan-treated *S. aureus*. To control for the possible effects of the fatty acids found in the soybean-based medium used in the assay described above, we evaluated the phenotypes in a fatty acid-free broth composed of 1% tryptone (22, 53). Kirby-Bauer disk diffusion assays were performed on tryptone agar augmented with EYP or not augmented. The addition of EYP to tryptone agar had a similar effect of reducing the triclosan-induced zone of inhibition in *S. aureus* described above (Fig. 1D). Fatty acid-free tryptone broth was used to test the ability of EYP (Fig. 1E) or human LDLs (Fig. 1F) to be utilized as sources of exogenous fatty acids when *S. aureus* is grown in the presence of triclosan. The addition of EYP or human LDLs to the fatty acid-free, tryptone medium also restored the growth of *S. aureus* cultured in the presence of triclosan. The ability of both chicken- and human-derived lipoprotein particles to reverse triclosan growth inhibition implies that lipoprotein particles serve as a source of exogenous fatty acids for *S. aureus*.

Host-derived lipoprotein particles are a source of exogenous fatty acids for *S. aureus*. Two possibilities may account for the ability of LDLs to rescue growth in the

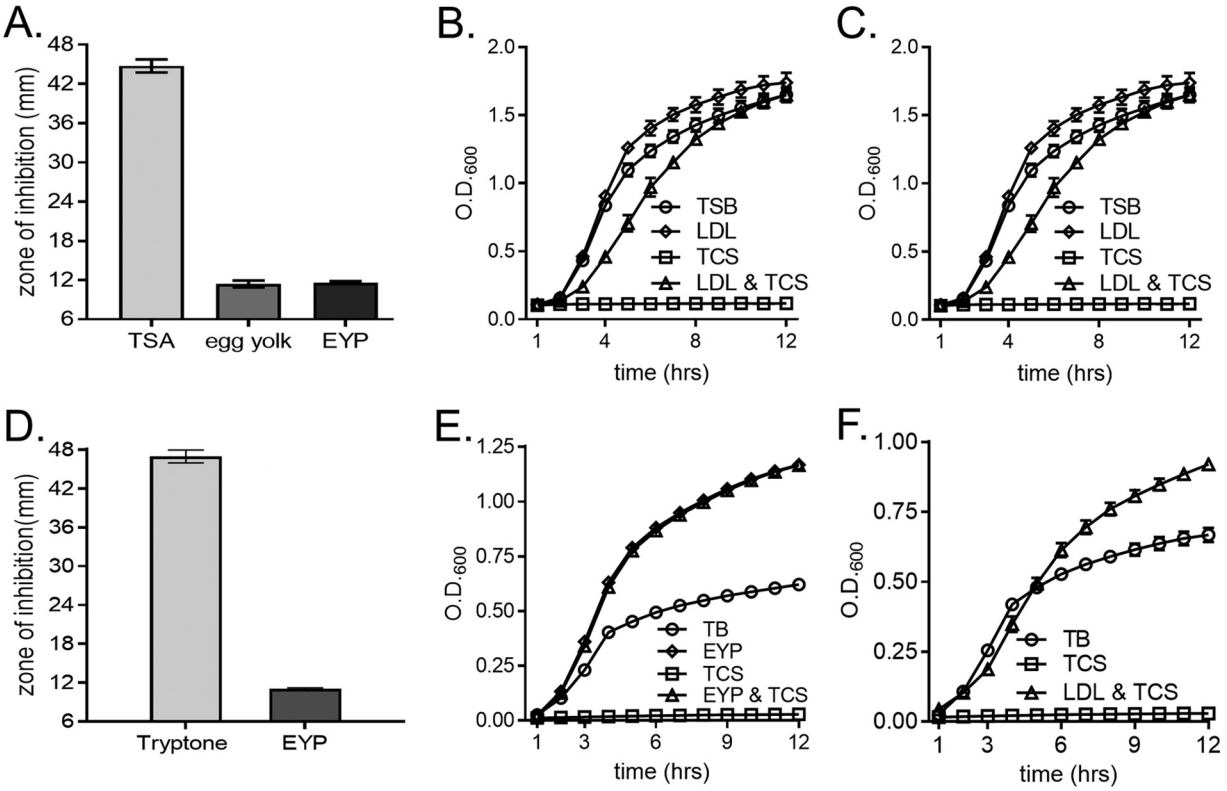

FIG 1 Lipoprotein particles protect *S. aureus* from FASII inhibition by triclosan. (A) *S. aureus* was plated as a lawn on tryptic soy agar (TSA) with the following supplements to determine triclosan sensitivity by Kirby-Bauer disk diffusion assays: no supplementation, 1% chicken egg yolk, and 1% chicken egg yolk plasma (EYP). The mean diameter of the zone of inhibition from four independent experiments is shown. Error bars represent the standard error of the mean. (B) The growth of *S. aureus* was monitored over time by measurement of the optical density (OD) in TSB under the following conditions: untreated, 1% EYP, 1 μM triclosan (TCS), or 1 μM triclosan with 1% EYP. The mean from six independent experiments is shown. Error bars represent the standard error of the mean. (C) The growth of *S. aureus* was monitored over time by measurement of the OD in TSB under the following conditions: untreated, 0.34 μg/μl purified human LDL, 1 μM triclosan, or 1 μM triclosan with 0.34 μg/μl purified human LDL. (D) *S. aureus* was plated as a lawn on 1% tryptone agar with or without EYP for Kirby-Bauer disk diffusion assays to determine triclosan sensitivity, as described in the legend to panel A. The mean diameter of the zone of inhibition from four independent experiments is shown. Error bars represent the standard error of the mean. (E) The growth of *S. aureus* was monitored over time by measurement of the OD in 1% tryptone broth under the following conditions: untreated, 1% EYP, 1 μM triclosan, or 1 μM triclosan with 1% EYP. The mean from four independent experiments is shown. Error bars represent the standard error of the mean. (F) The growth of *S. aureus* was monitored over time by measurement of the OD in 1% tryptone broth under the following conditions: untreated, 1 μM triclosan, or 1 μM triclosan with 0.34 μg/μl purified human LDL. The mean from four independent experiments is shown. Error bars represent the standard error of the mean.

presence of triclosan. First, a nonspecific triclosan-LDL interaction may sequester triclosan from the cells, resulting in rescued growth (22, 26). Alternatively, *S. aureus* actively incorporates LDL-derived fatty acids into its phospholipid bilayer, bypassing triclosan FASII inhibition. To distinguish between these two possibilities, we directly measured the amount of LDL-derived fatty acids that are incorporated into the staphylococcal membrane. *S. aureus* does not synthesize unsaturated fatty acids but can incorporate them into phosphatidylglycerol (PG), the major phospholipid present in the staphylococcal membrane (22, 54, 55). In the absence of exogenous fatty acids, the fatty acid moieties of *S. aureus* PG are predominantly saturated, consisting of 14 to 20 carbon atoms (22, 55). Consistent with this, saturated fatty acids of 15 and 17 carbons ($C_{15:0}$ and $C_{17:0}$) are the most common fatty acids comprising PG ($PG_{32:0}$) in *S. aureus* (22). Human LDL primarily contains $C_{16:0}$, $C_{18:0}$, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, and $C_{20:1}$ fatty acids esterified within triglycerides or to cholesterol (56, 57). Therefore, the unsaturated fatty acids $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, and $C_{20:1}$ serve as distinct molecular signatures of LDL-derived fatty acids. We employed an unbiased lipidomic analysis utilizing direct-infusion high-resolution/accurate mass spectrometry (MS) and tandem mass spectrometry to monitor the fatty acid profile of *S. aureus* PG. *S. aureus* was incubated in the presence or absence of human LDLs, and the PG profile of these cells was compared to that of cells cultured in 1%

tryptone broth (see Table S1 in the supplemental material). The PG profile of *S. aureus* grown in tryptone broth supplemented with human LDL was significantly different than the profile of cells cultured in the absence of LDL (Table 1). The altered PG profile included a large increase in the percentage of unsaturated PG (UPG) in the cell membrane (Fig. 2A). The unsaturated fatty acids that comprise the UPG are common within human LDLs, and we observed a 14.59-fold increase in these unsaturated fatty acids in the PG of cells cultured with human LDLs compared to those grown in tryptone broth alone (Fig. 2B). In keeping with these results, the four most abundant unsaturated fatty acids in human LDLs, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, and $C_{20:1}$, were all increased in *S. aureus* PG when the cells were cultured in the presence of human LDLs compared to when they were cultured without human LDLs (Table S1). In an independent experiment using TSB, we observed similar differences in the PG profiles of *S. aureus* cultures supplemented with LDL. Specifically, the relative abundance of all unsaturated fatty acids within the UPG was increased in cells cultured in the presence of human LDLs (Table S2), although the fatty acids from the soybean component of TSB (55, 58, 59) likely were also incorporated by *S. aureus*, making these results more difficult to interpret than the results of the analysis performed in fatty acid-free tryptone broth (55). Together, these data demonstrate that *S. aureus* incorporates unsaturated fatty acids present within human LDLs into the membrane PG.

Strains lacking triacylglycerol lipase activity demonstrate decreased incorporation of exogenous fatty acids into the cell membrane. We next sought to determine the mechanism by which *S. aureus* liberates free fatty acids from host LDLs. LDLs are composed of a structural protein and esterified fatty acids, such as triglycerides. Host lipases release fatty acids from the triglycerides of lipoprotein particles, which are then absorbed by the tissue (33). We hypothesized that *S. aureus* utilizes a protease or lipase to liberate fatty acids from LDLs. Thus, genetic inactivation of a protease or lipase would decrease triclosan resistance in the presence of LDLs. To test this, we examined the growth of *S. aureus* mutants inactivated for the protease aureolysin (*aur*) or V8 protease (*sspA*) and exposed to triclosan in the presence of lipoprotein particles (Fig. 3A). To assess the role of lipases in LDL-mediated triclosan resistance, the growth of mutants inactivated for the lipase encoded by *lip* or *geh* was quantified when the cells were treated with triclosan and lipoprotein particles (Fig. 3A). Kirby-Bauer disk diffusion assays were performed with wild-type (WT) *S. aureus* and protease or lipase mutants of *S. aureus* on tryptone agar plates supplemented with EYP. The mean diameter of the zone of inhibition produced by the *aur* and *sspA* protease mutants was approximately the same as that produced by the WT *S. aureus* control. Conversely, the *geh* mutant demonstrated an enhanced zone of inhibition, implying that this strain is more sensitive to triclosan upon supplementation with EYP. The *lip* mutant retained WT levels of triclosan resistance on plates supplemented with EYP. To determine if this phenotype extended to LDLs, we performed growth curves in fatty acid-free tryptone broth supplemented with human-derived LDLs in the presence of triclosan. Consistent with the Kirby-Bauer disk diffusion assay results (Fig. 3A), the capacity of human LDLs to rescue the growth of the *geh* mutant (Fig. 3B) exposed to triclosan was impaired, but the *lip* mutant (Fig. 3C) retained growth similar to that of the WT. A *geh lip* double mutant also demonstrated impaired growth compared to WT cultures supplemented with triclosan and human LDLs (Fig. 3D).

We hypothesized that the lipases liberate LDL-derived fatty acids. As Geh and Lip are secreted by the cell into the extracellular milieu (38), we monitored the ability of supernatants isolated from the WT and the *geh lip* double mutant to release free fatty acids from human LDLs using the unbiased lipidomic analysis described above. Human LDLs were mixed in tryptone broth to measure the background levels of free fatty acids. A low level of free fatty acids was present in the LDL preparation (Fig. S1). However, incubation of WT supernatants with human LDLs resulted in a 541.6-fold increase in total free fatty acids and an 829.0-fold increase in total free unsaturated fatty acids (Fig. 3E). This result supports the conclusion that an enzyme(s) secreted by *S. aureus* facilitates fatty acid release from human LDL. Moreover, the WT supernatant induced

TABLE 1 Unsaturated fatty acid profile of the WT and *S. aureus geh lip* grown in the presence of human LDLs

	WT cultured in tryptone broth			WT cultured in tryptone broth with human LDLs			*S. aureus geh lip* cultured in tryptone broth			*S. aureus geh lip* cultured in tryptone broth with human LDLs		
PG TC:TDB[a]	Normalized ion abundance/mg of cells	SD	Fatty acids[c]	Normalized ion abundance/mg of cells	SD	Fatty acids[c]	Normalized ion abundance/mg of cells	SD	Fatty acids[c]	Normalized ion abundance/mg of cells	SD	Fatty acids[c]
30:1	0.00	0.00	ND[b]	0.11	0.08	ND	0.13	0.06	ND	0.00	0.00	ND
31:1	1.96	0.19	16:1_15:0, 14:1_17:0, 12:1_19:0	7.46	0.38	16:1_15:0, 14:1_17:0, 12:1_19:0, 11:1_20:0	2.64	0.24	16:1_15:0, 14:1_17:0, 12:1_19:0	3.02	0.18	16:1_15:0, 14:1_17:0, 12:1_19:0, 11:1_20:0
32:1	1.49	0.07	ND	7.79	0.58	17:1_15:0, 16:1_16:0, 18:1_14:0	1.99	0.29	17:1_15:0, 15:1_17:0, 13:1_19:0	2.18	0.10	17:1_15:0, 16:1_16:0
33:1	12.76	1.01	18:1_15:0	214.05	19.82	18:1_15:0	14.19	0.59	18:1_15:0	28.02	1.43	18:1_15:0
34:1	0.00	0.00	ND	16.58	1.53	18:1_16:0, 16:1_18:0	0.00	0.00	ND	0.00	0.00	ND
34:2	0.00	0.00	ND	5.04	0.37	16:1_18:1, 18:2_16:0	0.00	0.00	ND	2.00	0.25	16:1_18:1, 18:2_16:0
35:1	17.07	1.75	20:1_15:0, 19:1_16:0	61.86	5.26	20:1_15:0, 18:1_17:0, 19:1_16:0	19.16	0.22	20:1_15:0, 19:1_16:0	14.66	0.46	20:1_15:0, 19:1_16:0, 18:1_17:0
35:2	0.00	0.00	ND	8.04	0.50	20:2_15:0, 18:2_17:0, 16:1_19:1	0.03	0.01	ND	6.04	0.23	20:2_15:0, 18:2_17:0
35:4	0.00	0.00	ND	1.46	0.26	20:4_15:0, 17:2_18:2, 20:3_15:1	0.00	0.00	ND	20.13	0.60	20:4_15:0
35:5	0.00	0.00	ND	0.00	0.00	ND	0.00	0.00	ND	1.27	0.15	20:5_15:0, 20:4_15:1
36:2	0.00	0.00	ND	46.15	4.14	18:1_18:1, 18:2_18:0	0.00	0.00	ND	0.39	0.16	18:2_18:0, 18:1_18:1
36:3	0.11	0.05	ND	17.05	1.56	18:1_18:2, 20:3_16:0	0.00	0.00	ND	0.20	0.10	18:2_18:1
36:4	0.00	0.00	ND	2.34	0.35	18:2_18:2, 16:0_20:4, 18:3_18:1	0.00	0.00	ND	0.25	0.06	18:2_18:2, 20:3_16:1, 20:4_16:0
37:2	0.00	0.00	ND	1.85	0.11	19:0_18:2	0.00	0.00	ND	0.38	0.15	19:0_18:2
37:4	0.00	0.00	ND	0.00	0.00	ND	0.00	0.00	ND	0.91	0.05	20:4_17:0

[a]Detected as $[M\text{-}H]^-$ ions. TC, total chain length; TDB, total number of double bonds.

[b]ND, not determined.

[c]Fatty acids are listed in order of isomer abundance. An underscore between fatty acid designations indicates that each fatty acid may be present in either the SN1 or SN2 position, as tandem mass spectrometry data alone cannot rule out the possibility that lipid species exist as a mixture of positional isomers (85).

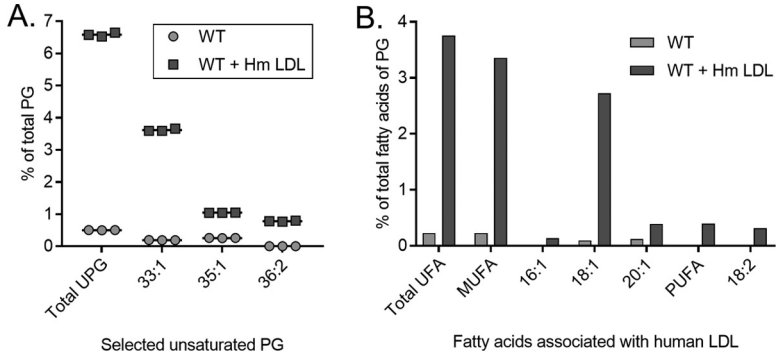

FIG 2 Human low-density lipoprotein (Hm LDL) particles are a source of exogenous fatty acids for synthesis of *S. aureus* phosphatidylglycerol. (A) Percentage of unsaturated phosphatidylglycerol (UPG) in comparison to the amount of total membrane PG of *S. aureus* grown in the presence (WT + Hm LDL) or absence (WT) of human LDLs. (B) Unsaturated fatty acid profile of membrane PG of *S. aureus* grown with (WT + Hm LDL) or without (WT) human LDLs plotted as a percentage of the amount of total PG fatty acids. UFA, MUFA, and PUFA, unsaturated, monounsaturated, and polyunsaturated fatty acids, respectively.

the liberation of the LDL-derived unsaturated fatty acids $C_{16:1}$, $C_{18:1}$, and $C_{18:2}$, which had fold increases of 1,733.4, 1,074.9, and 1,226.7, respectively (Fig. 3E). These data are consistent with the results presented in Fig. 2A, demonstrating that culturing *S. aureus* in the presence of LDL increases the abundance of $C_{16:1}$, $C_{18:1}$, and $C_{18:2}$ within UPG (Fig. 2A). The release of free fatty acids from human LDLs was lipase dependent, as supernatants isolated from the *geh lip* mutant demonstrated a significant reduction in the quantity of total free fatty acids after incubation with human LDLs (Fig. 3E). The amounts of the unsaturated fatty acids $C_{16:1}$, $C_{18:1}$, and $C_{18:2}$ were markedly decreased compared to those in the WT supernatants, as denoted by 704.4-, 292.0-, and 259.8-fold reductions, respectively (Fig. 3E).

To determine if the decreased liberation of fatty acids from human LDLs leads to a reduction in LDL-derived fatty acids within the *geh lip* mutant PG, we performed lipodomics on the *geh lip* mutant cultured in the presence of human LDLs. In the absence of LDLs, WT and *geh lip* mutant cells had similar PG profiles (Table 1). However, upon exposure to human LDLs, the amount of UPG for the *geh lip* mutant was reduced 7.74-fold compared to that for the WT (Fig. 3F). In keeping with this, the levels of total unsaturated fatty acids in the membrane were reduced 5.46-fold compared to those in the membrane of the WT grown with LDLs (Fig. 3G). These data demonstrate that exogenous fatty acid source incorporation from a complex fatty source is impaired in the *geh lip* mutant. However, the mutant does retain some ability to incorporate exogenous fatty acids from LDLs into its PG. In keeping with this, we observed increases in the UPG species $PG_{35:4}$ and $PG_{35:5}$ in the *geh lip* mutant (Table S1). These species contain $C_{20:4}$ and $C_{20:5}$ unsaturated fatty acids. The incorporation of these unsaturated fatty acids in the *geh lip* mutant provides an explanation for why the *geh lip* mutant retains LDL-dependent triclosan resistance. The relatively small amount of free fatty acids present within the LDL preparation likely accounts for some of the LDL-dependent triclosan growth as well.

The methicillin-sensitive *S. aureus* strain Newman (NM) is lipase negative due to a prophage insertion that disrupts *geh* (60). Having established that the loss of *geh* expression in the laboratory-derived USA300 strain alters the ability of *S. aureus* to exploit lipoprotein particles as a source of exogenous fatty acids, we reasoned that the prophage-induced loss of *geh* expression in NM would similarly impair this strain's ability to utilize lipoprotein particles for triclosan protection. To assess this, WT NM and TB4, a previously described NM variant in which Geh activity was restored via prophage excision (60), were exposed to triclosan in the presence of lipoprotein particles. Kirby-Bauer disk diffusion assays were completed with WT NM and TB4 on tryptone agar plates supplemented with EYP (Fig. 4A). The mean diameter of the zone of inhibition for NM was 5.4 mm larger than that for USA300, while the mean diameter for

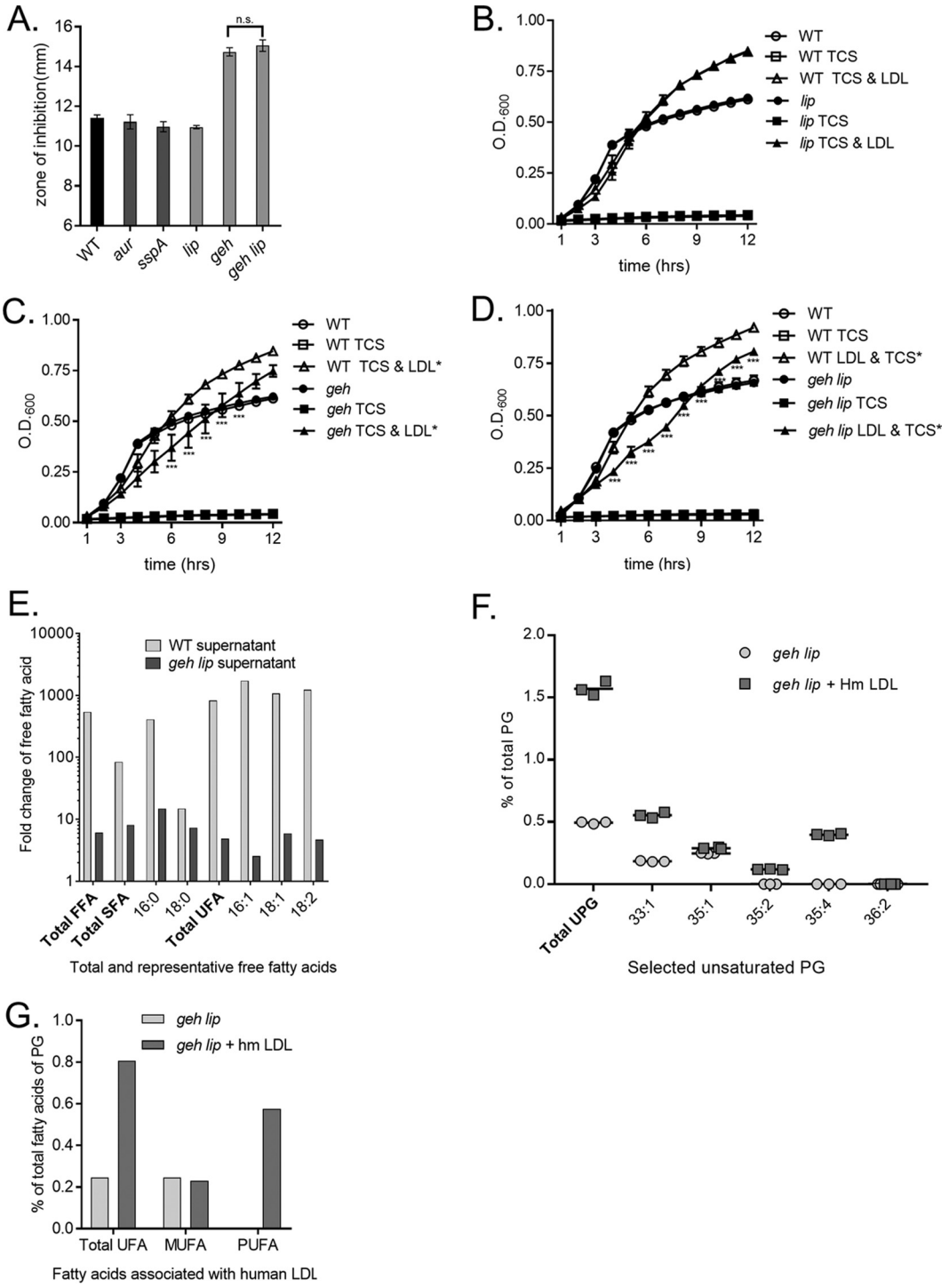

FIG 3 The *S. aureus* lipase, encoded by *geh*, is required for full triclosan protection by lipoprotein particles. (A) The WT and protease (*aur* and *sspA*) and lipase (*lip*, *geh*, and *lip geh*) mutants were plated from overnight cultures as a lawn on 1% tryptone agar with or without 1% EYP. A triclosan-impregnated disk was placed on top of the agar. The mean diameter of the zone of inhibition from four independent experiments is shown. All error bars represent the standard error of the mean. (B to D) The growth of the WT and an isogenic *geh* transposon mutant (B), an isogenic *lip* transposon mutant (C), and an isogenic *geh lip* transposon mutant (D) was monitored over time by determination of the optical density (OD). Cells were grown in 1% tryptone broth under the following conditions: untreated, 1 μM triclosan (TCS), or 1 μM triclosan with 0.34 μg/μl purified human LDL. The mean results from four independent experiments are shown. ***, $P < 0.005$ by two-way ANOVA between the WT plus triclosan and LDL versus the *geh* or *geh lip* mutant plus triclosan and LDL; n.s., not statistically significant. All error bars represent the standard error of the mean. (E) The WT and the *geh lip* double mutant supernatants were incubated with human LDLs in triplicate. Free fatty acids (FFA) were detected by direct-infusion high-resolution/accurate mass spectrometry and tandem mass spectrometry, and the fold change calculated was based on the normalized number of ions per milligram. The

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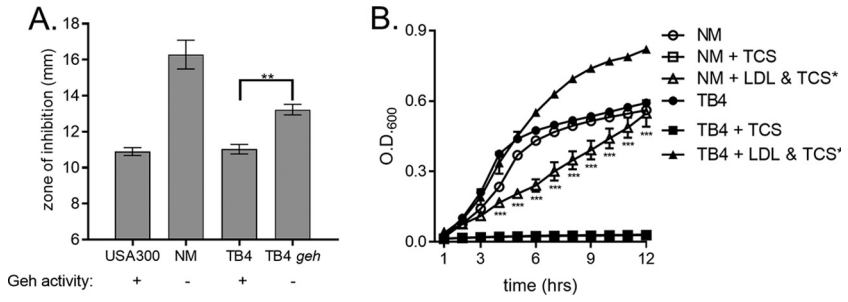

FIG 4 Lipoprotein particle protection from triclosan is reduced in lipase-negative strain Newman. (A) USA300, Newman (NM), TB4, and TB4 *geh* were plated as a lawn on 1% tryptone agar with or without EYP. A triclosan-impregnated disk was placed on top of the agar. The mean from four independent experiments is shown. Error bars represent the standard error of the mean. **, $P = 0.0015$ by Student's *t* test. (B) The growth of NM and TB4 was monitored over time by measurement of the OD in 1% tryptone broth under the following conditions: untreated, 1 μM triclosan (TCS), or 1 μM triclosan with 0.34 μg/μl purified human LDL. The mean results from four independent experiments are shown. ***, $P < 0.005$ by two-way ANOVA between NM plus triclosan and LDL versus TB4 plus triclosan and LDL. Error bars represent the standard error of the mean.

the lipase-positive TB4 was not significantly different from that for USA300. To evaluate the contribution of *geh* to the difference observed between WT and TB4, we transduced a transposon-disrupted allele of *geh* into TB4, reducing its lipase activity. This resulted in a statistically significant increase in the triclosan-induced zone of inhibition in the TB4 *geh* mutant compared to that in TB4. Growth curves were conducted with NM and TB4 using purified human LDLs as the fatty acid source. Triclosan treatment completely suppressed the growth of both NM and TB4. However, NM growth was suppressed when the strain was cultured in the presence of both triclosan and human LDL (Fig. 4B). Conversely, supplementation of TB4 cells with human LDL resulted in a restoration of growth in the presence of triclosan.

The capacity of exogenous fatty acids to increase *S. aureus* triclosan resistance is dependent on the fatty acid kinase FakA. We observed that *S. aureus* incorporates host-derived fatty acids present in LDL into its membrane PG and that the major triacylglycerol lipase, encoded by *geh*, plays a role in this incorporation. Elegant studies have demonstrated that the fatty acid kinase FakA is required for the incorporation of exogenous fatty acids into the staphylococcal membrane (28, 29, 61). In keeping with this, we surmised that the fatty acid kinase FakA facilitates incorporation of the LDL-derived fatty acids into membrane PG. Inactivation of *fakA* has been proposed to increase triclosan sensitivity in *S. aureus*, despite being cultured with a source of exogenous fatty acid (20), but this has yet to be experimentally confirmed. Therefore, we sought to establish that *fakA* is necessary for exogenous fatty acid protection from triclosan in *S. aureus*. To test this, we analyzed the growth of a previously described *S. aureus* (USA300) *fakA* deletion mutant (the Δ*fakA* mutant) exposed to triclosan in the presence and absence of exogenous fatty acids (62). Kirby-Bauer disk diffusion assays were performed with WT *S. aureus* and an *S. aureus* Δ*fakA* mutant on agar plates with or without a mixture of the fatty acids palmitic acid, oleic acid, and myristic acid (20). The zone of inhibition observed for the Δ*fakA* mutant grown on TSA was slightly larger than that observed for WT *S. aureus* grown on the same agar medium (Fig. 5A). We attributed this difference to the fatty acids present in the soybean meal component of TSA being utilized by the WT but not the Δ*fakA* mutant (58, 63). The mean diameter of the zone of inhibition produced by the Δ*fakA* mutant on the fatty acid-supplemented

FIG 3 Legend (Continued)
fold change represents the amount of free fatty acids present in supernatant-treated samples compared to the amount of free fatty acids in the LDL preparation diluted in 1% tryptone broth. (F) Percentage of unsaturated PG (UPG) in comparison to the amount of total membrane PG of *S. aureus* grown in the presence or absence of human LDLs. (G) Unsaturated fatty acid profile of membrane PG of *S. aureus* grown with or without human LDLs plotted as a percentage of the amount of total PG fatty acids. SFA, UFA, MUFA, and PUFA, saturated, unsaturated, monounsaturated, and polyunsaturated fatty acids, respectively.

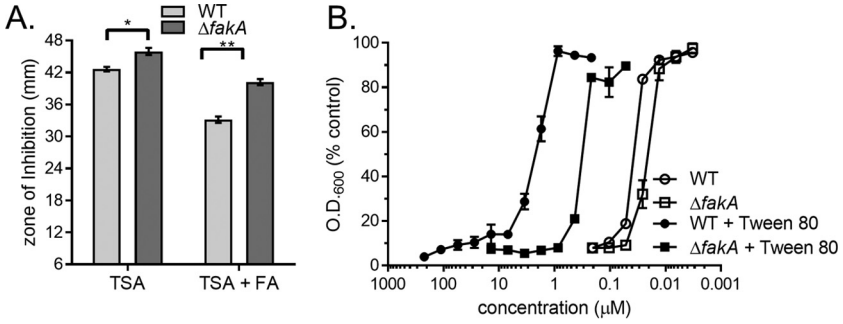

FIG 5 *fakA* mediates exogenous fatty acid bypass of triclosan in *S. aureus*. (A) WT *S. aureus* and an isogenic Δ*fakA* mutant were plated from overnight cultures separately as a lawn on TSA or TSA supplemented with a fatty acid (FA) mixture consisting of $C_{14:0}$, $C_{16:0}$, and $C_{18:1}$. The mean diameter of the zone of inhibition from six independent experiments is shown. Error bars represent the standard error of the mean. Statistical significance was calculated by two-way ANOVA with a Tukey *post hoc* test for multiple comparisons; *, $P = 0.0029$; **, $P < 0.0001$. (B) The antimicrobial activities of triclosan against WT *S. aureus* and the Δ*fakA* mutant with and without 0.1% Tween 80 were compared. Cells were then suspended by pipetting, and the OD at 600 nm was measured. Results are presented as the percentage of the value for the untreated control cells. The mean from four independent experiments is shown. Error bars represent the standard error of the mean.

agar was significantly larger than that produced by the WT (Fig. 5A). To further refine the role of *fakA* in the fatty acid-mediated tolerance of *S. aureus* to triclosan, we determined the half-maximal inhibitory concentration (IC_{50}) of WT and the Δ*fakA* mutant cells to triclosan in the presence of an exogenous fatty acid source, Tween 80. Previous studies have shown that Tween 80 is a viable source of exogenous fatty acids that decreases sensitivity to FASII inhibitors (21, 26, 64). Compared to cells cultured in the absence of fatty acids, the IC_{50} of WT *S. aureus* grown in the presence of Tween 80 demonstrated a 54.0-fold decrease in triclosan sensitivity (Fig. 5B). Under the same conditions, the Δ*fakA* mutant demonstrated only a 9.39-fold reduction in the IC_{50} between Tween 80-supplemented TSB and TSB lacking supplementation. This difference is a substantially smaller change in triclosan sensitivity compared to that for the WT. Taken together, these data demonstrate that genetic inactivation of FakA impairs the protective effect of exogenous fatty acids shown in response to the FASII inhibitor triclosan.

***S. aureus* exogenous fatty acid incorporation from lipoprotein particles is *fakA* dependent.** Having established that *fakA* is required for the fatty acid-dependent tolerance of triclosan, we next wanted to determine if *fakA* is also required for the LDL-dependent tolerance of triclosan. To test this, we assessed the sensitivity of the Δ*fakA* mutant to triclosan using three different assays. First, Kirby-Bauer disk diffusion assays revealed that the sensitivity of the Δ*fakA* mutant to triclosan is enhanced compared to that of the WT when the strains were grown on tryptone agar supplemented with lipoprotein particle-rich EYP (Fig. 6A). Second, we determined the IC_{50} of the WT and the Δ*fakA* mutant exposed to triclosan and supplemented with chicken egg yolk LDLs in TSB. The IC_{50}s of triclosan for the Δ*fakA* mutant in the presence of egg yolk LDLs was 0.10 μM, while that for the WT was 0.29 μM (Fig. 6B). These results indicate that the Δ*fakA* mutant is impaired for utilizing LDLs as a source of fatty acids. Finally, growth curves were performed with the Δ*fakA* mutant using 0.34 μg/μl purified human LDLs as the fatty acid supplement in 1% tryptone broth. Triclosan treatment suppressed the growth of the Δ*fakA* mutant, despite supplementation with human LDL (Fig. 6C). Conversely, supplementation of WT cells with human LDL suppressed triclosan growth inhibition (Fig. 6C). In total, the inability of lipoprotein particles to protect the Δ*fakA* mutant from triclosan demonstrates that host-derived lipoprotein particles are exploited as a source of fatty acids for *S. aureus*.

***fakA* is necessary for the generation of triclosan-resistant mutants of *S. aureus*.** The generation of spontaneous triclosan-resistant *S. aureus* fatty acid auxotrophs through exposure to FASII inhibitors on media supplemented with free fatty acids has

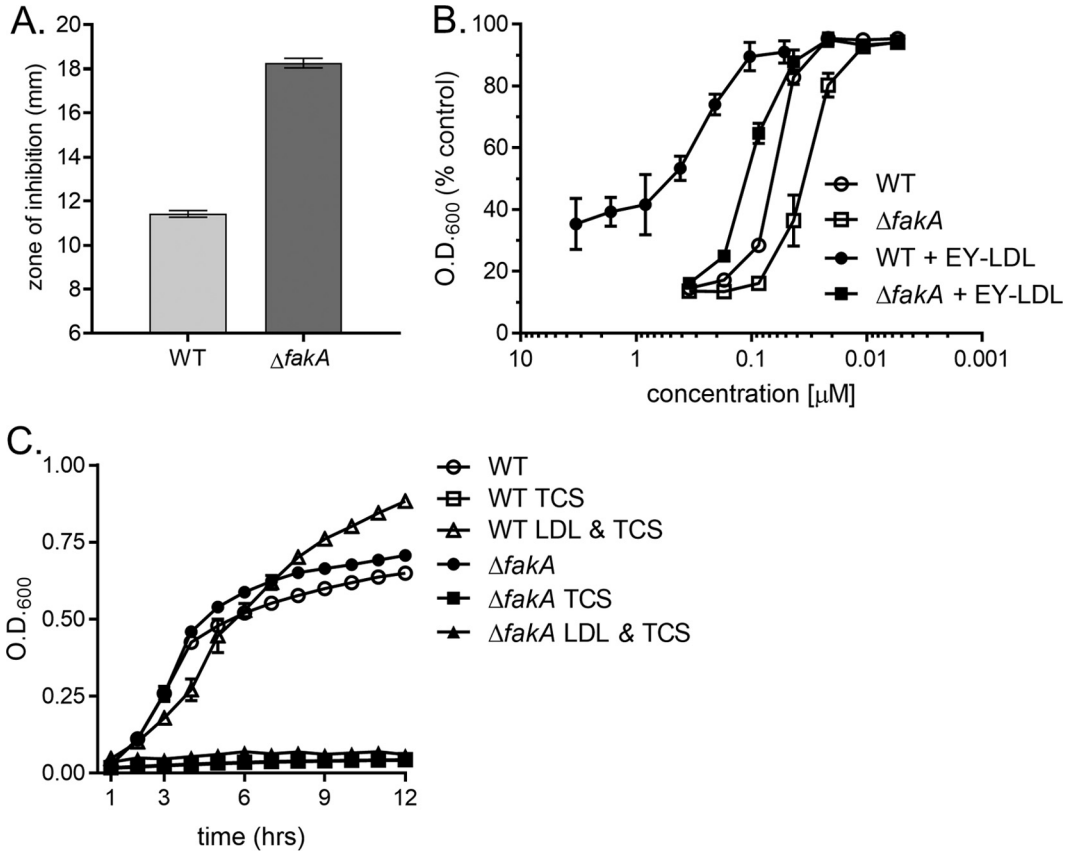

FIG 6 *fakA* is required for lipoprotein particle-dependent protection from triclosan. (A) The WT and the isogenic Δ*fakA* mutant were plated from overnight cultures as a lawn on TSA or TSA supplemented with 1% EYP for Kirby-Bauer disk diffusion assays to determine triclosan sensitivity. The mean diameter of the zone of inhibition from four independent experiments is shown. All error bars represent the standard error of the mean. (B) The antimicrobial activities of triclosan against the WT and the Δ*fakA* mutant with and without lipoprotein particles were compared by culturing cells in the presence of 0.1% purified chicken egg yolk LDL (EY-LDL) containing increasing concentrations of triclosan. The cells were then suspended, and the OD_{600} was measured. Results are presented as the percentage of the value for untreated control cells. The mean results from four independent experiments are shown. All error bars represent the standard error of the mean. (C) The growth of the WT and an isogenic Δ*fakA* mutant was monitored over time by measurement of the OD_{600} in 1% tryptone broth under the following conditions: untreated, 1 μM triclosan (TCS), or 1 μM triclosan with 0.34 μg/μl purified human LDL. The mean results from three independent experiments are shown. All error bars represent the standard error of the mean.

previously been described by Morvan et al. (20). The mutations in the majority of these mutants map to the genes of the FASII initiation pathway (20). To determine if FakA is required for the generation of triclosan-resistant mutants, we plated WT and Δ*fakA* mutant cells on TSA with or without the fatty acid supplement (palmitic acid, oleic acid, and myristic acid), and a disk infused with triclosan was placed on the agar (Fig. 7). After 24 h of incubation, the number of resistant colonies that grew within the triclosan-induced zone of inhibition on fatty acid-supplemented agar generated by the WT was significantly greater than the number that grew within the zone of inhibition generated by the *fakA* mutant. After an additional 24 h of incubation, WT *S. aureus* continued to generate triclosan-resistant colonies on both supplemented agar and agar lacking supplementation, but the number of resistant colonies generated by the Δ*fakA* mutant was significantly reduced. These results indicate that the fatty acid-dependent generation of triclosan-resistant mutants is facilitated by FakA.

Human LDLs support the growth of fatty acid auxotrophs of *S. aureus*. We sought to determine if lipoprotein particles satisfy the fatty acid requirement of *S. aureus* fatty acid auxotrophs. These studies allowed us to assess the ability of *S. aureus* to incorporate fatty acids from LDLs without relying on triclosan to inhibit endogenous fatty acid synthesis. The generation of *S. aureus* fatty acid auxotrophs through mutation

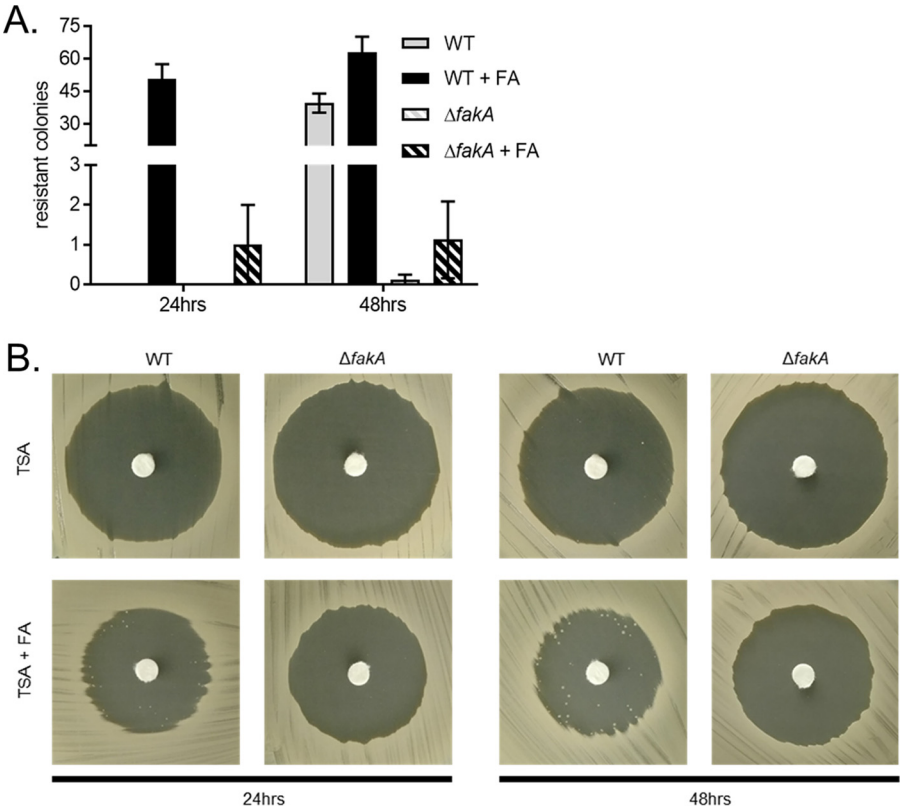

FIG 7 Generation of triclosan-resistant mutants in the presence of exogenous fatty acids is *fakA* dependent. (A) WT *S. aureus* and the isogenic Δ*fakA* mutant were plated from overnight cultures as a lawn on TSA or TSA containing the fatty acid supplement, and a triclosan-impregnated disk was placed on top of the agar. Isolated colonies that grew within the zone of inhibition were counted after 1 and 2 days of growth. The mean results from four independent experiments are shown. Error bars represent the standard error of the mean. (B) Representative images of individual treatments are shown after 24 h and 48 h of incubation.

in the genes of the FASII initiation pathway has previously been described (20, 23, 24). To ascertain if fatty acid auxotrophs are capable of utilizing human LDLs as a source of fatty acids, three resistant mutants from three independent trials were isolated from the zone of inhibition in the assays whose results are shown in Fig. 7. We sequenced the FASII initiation genes *fabD*, *accA*, *accB*, *accC*, and *accD* of the auxotrophs and mapped inactivating mutations in at least one of these genes in five out of nine of the strains (Table S3). In keeping with the fact that the mutants require an exogenous source of fatty acids, the strains demonstrated little to no growth on TSA but were capable of growing on plates supplemented with free fatty acids or EYP (Fig. S2). Using these auxotrophs, we performed triclosan Kirby-Bauer disk diffusion assays on TSA containing either the fatty acid supplement or 1% EYP. The mean diameter of the zone of inhibition generated by the auxotrophs was significantly smaller than that generated by the WT when the medium was supplemented with fatty acids (Fig. 8A). Additionally, the zone of inhibition generated by the fatty acid auxotrophs plated on agar supplemented with EYP was below our limit of detection of 6 mm (Fig. 8A). These data suggest that the fatty acid auxotrophs utilize lipoprotein particle-derived fatty acids to support their growth in the presence of triclosan. To determine if lipoprotein particles support the growth of the fatty acid auxotrophs, growth curves were performed using purified chicken LDLs as a source of exogenous fatty acids in fatty acid-free tryptone broth. Medium lacking chicken LDL supplementation did not support growth (Fig. 8B), whereas the auxotrophs grew upon medium with chicken LDL supplementation (Fig. 8C). Finally, we sought to extend our observation using a more relevant source of fatty acids, purified human LDLs. Human LDLs also supported the growth of the fatty acid

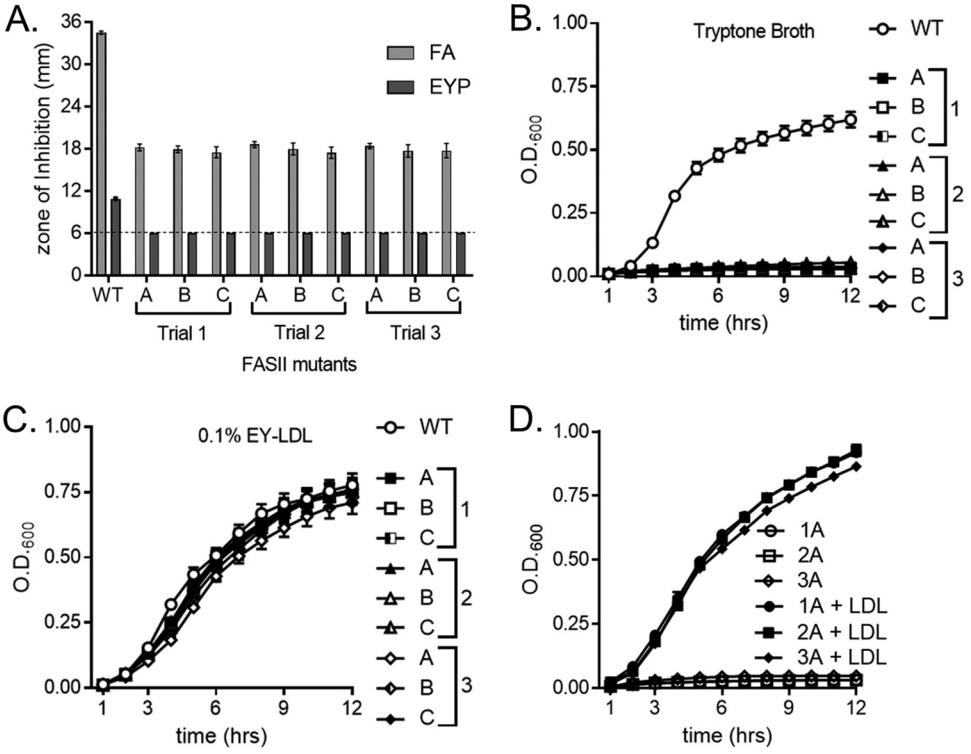

FIG 8 LDL supplements the growth of triclosan-resistant, fatty acid auxotrophs. (A) Triclosan-resistant fatty acid auxotrophs were plated as a lawn on TSA with the following supplement: fatty acid mixture or 1% EYP. A triclosan-impregnated disk was placed on top of the agar. The dashed line denotes the limit of detection of 6 mm. The mean diameter of the zone of inhibition from three independent experiments is shown. All error bars represent the standard error of the mean. (B) The growth of the triclosan-resistant fatty acid auxotrophs in 1% tryptone broth was monitored over time by measurement of the OD. (C) The growth of the triclosan-resistant fatty acid auxotrophs in 1% tryptone broth with 0.1% purified egg yolk LDL (EY-LDL) was monitored over time by measurement of the OD. Squares represent mutants isolated in trial 1, triangles denote mutants isolated in trial 2, and diamonds depict mutants isolated in trial 3. The mean results from four independent experiments are shown. All error bars represent the standard error of the mean. (D) The growth of a representative triclosan-resistant fatty acid auxotroph from each trial was monitored over time by measurement of the OD_{600}. Cells were grown in 1% tryptone broth under the following conditions: untreated or supplementation with 0.34 μg/μl purified human LDL. The mean results from three independent experiments are shown. All error bars represent the standard error of the mean.

auxotrophs in tryptone broth, while medium lacking supplementation could not (Fig. 8D). These findings show that a human lipoprotein particle, LDL, provides *S. aureus* with the fatty acids necessary to bypass FASII inhibition.

Lipoprotein particles protect clinical isolates of *S. aureus* from triclosan inhibition. We next sought to discern if the lipoprotein particle-dependent protection that we observed in laboratory-derived strains of *S. aureus* extended to clinical isolates. We characterized three lipase-negative isolates from a single cystic fibrosis (CF) patient across multiple time points, while isolates from the remaining six CF patients were all lipase positive (data not shown). Next, we performed Kirby-Bauer disk diffusion assays on EYP-supplemented tryptone agar utilizing lipase-positive isolates from three different CF patients and the three lipase-negative isolates from the single CF patient. The three lipase-negative strains were isolated at different times, and they also varied in their oxacillin sensitivity, implying that these were not clonal isolates. The zones of inhibition observed for the three lipase-positive isolates were nearly identical to those observed for the laboratory-derived USA300 strain (Fig. 9A). In contrast, the three lipase-negative isolates all produced a zone of inhibition larger than that produced by USA300 or any of the lipase-positive isolates. Growth curves were performed with a selected lipase-positive isolate and lipase-negative isolate, and these strains grew similarly in tryptone broth (Fig. 9B). Consistent with our previous lipase results (Fig. 3 and 4), the lipase-negative isolate demonstrated reduced growth compared to the

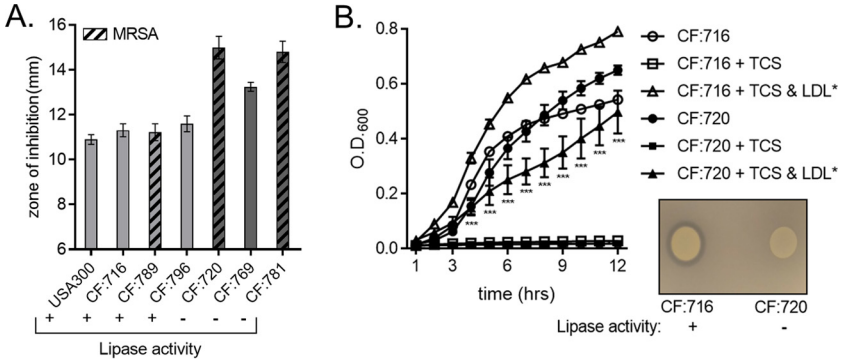

FIG 9 Clinical isolates of *S. aureus* demonstrate a variable capacity to utilize lipoprotein particles as sources of exogenous fatty acids. (A) Three lipase-positive and three lipase-negative clinical isolates of *S. aureus*, together with USA300 as a control, were plated as a lawn on 1% tryptone agar supplemented with EYP or not supplemented. A triclosan-impregnated disk was placed on top of the agar. The mean diameter of the zone of inhibition from four independent experiments is shown. Error bars represent the standard error of the mean. (B) The growth of a selected lipase-positive and a lipase-negative clinical isolate of *S. aureus* was monitored over time by measurement of the OD_{600}. Cells were grown in 1% tryptone broth under the following conditions: untreated, 1 μM triclosan (TCS), or 1 μM triclosan with 0.34 μg/μl purified human LDL. The mean results from four independent experiments are shown. ***, $P < 0.005$ by two-way ANOVA between CF:716 plus triclosan and LDL versus CF:720 plus triclosan and LDL. The mean from four independent experiments is shown. Error bars represent the standard error of the mean. (Inset) Overnight cultures of the selected lipase-positive and -negative clinical isolates were normalized to an OD_{600} of 0.5, and then 5 μl of these cultures was spotted onto a 1% tryptone agar plate containing EYP to determine lipase activity.

lipase-positive isolate. Cumulatively, the inability of lipoprotein particles to protect *S. aureus* strains with reduced lipase activity from triclosan supports a model whereby staphylococcal lipases, such as Geh, liberate esterified fatty acids from host lipoprotein particles. The freed fatty acids are then utilized by *S. aureus* to synthesize membrane phospholipids in a FakA-dependent fashion (Fig. S3).

DISCUSSION

Antimicrobial-resistant pathogens are a public health crisis, with one of the most prevalent being *S. aureus* (65). This fact has precipitated research focused on developing inhibitors against the bacterial *de novo* fatty acid synthesis pathway, FASII (4, 5). However, *S. aureus* and many other Gram-positive pathogens bypass FASII inhibition via the incorporation of exogenous fatty acids into their membrane phospholipids (21, 27, 29). Several studies have established that the sensitivity of *S. aureus* to triclosan and other FASII inhibitors decreases when exogenous fatty acids are available (20–22, 26, 44). Thus, the therapeutic potential of FASII inhibitors for the treatment of *S. aureus* infection is uncertain. However, identifying the sources of host-derived fatty acids utilized by *S. aureus* may lead to strategies that improve the efficacy of FASII inhibitors.

In the current work, we monitored the capacity of host lipoprotein particles to serve as reservoirs of exogenous fatty acids for *S. aureus*. Through the combined approaches of chemical and genetic inhibition of FASII, together with mass spectrometry-based analysis of the fatty acid composition of the bacterial membrane, we determined that human LDLs are a viable source of exogenous fatty acids for laboratory-derived and clinical isolates of *S. aureus*. LDLs are abundant in multiple types of fatty acids that are esterified as phospholipids, cholesterol esters, and triglycerides (33). LDLs and the other lipoprotein particles are prevalent in the serum fraction of the blood and account for the majority of the host's fatty acids (30–32, 66). Several studies have noted that the MIC of various FASII inhibitors for *S. aureus* increases when serum is added to the FASII inhibitor-treated cultures (21, 22, 67–69). Additionally, it has been demonstrated that the culture of *S. aureus* with serum leads to incorporation of host-derived fatty acids into the cell membrane phospholipids (55). Given this, we asked if the observed increase in triclosan tolerance is due to incorporation of LDL-derived fatty acids into the membrane phospholipids of *S. aureus*. Using mass spectrometry, we established that

the fatty acid profile of the membrane phospholipids is altered in *S. aureus* cultured in the presence of human LDLs. Specifically, the staphylococcal membrane included PG with $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, and other unsaturated fatty acids. *S. aureus* is not capable of synthesizing unsaturated fatty acids, but they are abundant in human lipoprotein particles (22). Consistent with these facts, we conclude that *S. aureus* liberates fatty acids from the LDL particle for incorporation into its phospholipids. We observed that unsaturated fatty acids that are commonly integrated into the cell membrane after exposure to human LDLs were among the free fatty acids with the largest fold increase and were some of the most abundant free fatty acids liberated after the human LDLs were incubated with *S. aureus* supernatant. These results are consistent with previous reports showing that the serum fraction of blood serves as a source of exogenous fatty acids for *S. aureus* phospholipid synthesis (55). Additional support for *S. aureus* modification of fatty acids from LDLs is that the levels of free fatty acids, including $C_{16:1}$, $C_{18:1}$, and $C_{18:2}$, within the commercial preparation of human LDLs was minimal. Importantly, significant increases in the levels of free fatty acids were observed when incubating culture supernatant with these LDLs, including individual increases of over 1,000-fold for $C_{16:1}$, $C_{18:1}$, and $C_{18:2}$. Importantly, the changes that we observed were dependent on the expression of staphylococcal lipases (Fig. 3E), and these data are consistent with those from multiple growth analyses demonstrating that lipase-deficient strains are impaired for LDL protection from triclosan (Fig. 3, 4, and 9). Therefore, we posit that accumulation of LDL-derived fatty acids in *S. aureus* PG species is primarily due to modification and incorporation of the fatty acids from human LDL. Genetic inactivation of the fatty acid kinase FakA also decreased the ability of *S. aureus* to resist FASII inhibition in the presence of LDLs, lending further support to our model. Together, these results demonstrate that *S. aureus* utilizes a complex, host-derived source of fatty acids for incorporation into the membrane phospholipid.

An ongoing debate in regard to *S. aureus* incorporation of exogenous fatty acids is the proposed requirement for endogenous fatty acid synthesis. Phospholipids comprise two fatty acid moieties, each occupying a specific position, sn1 or sn2, in the glycerol backbone. Elegant biochemical studies have led to the suggestion that fatty acids synthesized by the FASII pathway must occupy the sn2 position (22, 23, 27). However, the generation of fatty acid auxotrophs via the inhibition of endogenous fatty acid synthesis reported herein and by others brings into question the requirement of endogenous fatty acids populating the sn2 position (20, 22, 23). These mutants fail to grow in the absence of a source of fatty acids; therefore, it is likely that exogenous fatty acids populate both the sn1 and sn2 positions. Unfortunately, a limitation of our analysis is that we cannot discern which position the LDL-derived fatty acid populates. However, we observed PG species that contained only host-derived unsaturated fatty acids when analyzing the fatty acid content of *S. aureus* cultured with human LDLs, implying that an exogenous fatty acid can populate the sn2 position (see Table S4 in the supplemental material). Our finding that exogenous fatty acids can be placed in either position of PG is consistent with several recent reports, although the mechanism for this remains elusive (20, 24). Clearly, additional experimentation is required to resolve these conflicting models. However, the results reported herein provide additional evidence that (i) fatty acid auxotrophs can be generated by selecting for FASII inhibitor-resistant mutants, (ii) generation of these mutant auxotrophs is dependent on FakA activity, and (iii) human LDLs serve as a source of fatty acids for these auxotrophs.

Fatty acid auxotrophs of *S. aureus* have been previously reported and primarily result from mutations in *accABCD* and *fabD* (20, 22–24). Therefore, we sought to demonstrate that LDLs can supply the fatty acids required to support the growth of a fatty acid auxotroph. We generated fatty acid auxotrophs of *S. aureus* following a previously described method (20). Similarly, the mutants that we isolated grew only upon supplementation with free fatty acids. Importantly, supplementation of the growth medium with LDLs also supported the growth of the fatty acid auxotrophs, demonstrating that LDLs can be utilized as an exogenous source of fatty acids. Sequencing of the *fabD*, *accA*, *accB*, *accC*, and *accD* open reading frames of the

auxotrophs identified at least one mutation in the FASII initiation pathway for five of the nine mutants (Table S3). Presumably, the remaining four fatty acid auxotrophs have mutations outside the coding regions of the FASII initiation genes that limit their expression, and genome sequencing would more conclusively locate these mutations. Nonetheless, it is clear that the growth of these mutants is dependent on an exogenous source of fatty acids and that human LDLs satisfy this requirement. These findings are consistent with the supposition that *S. aureus* liberates LDL-derived fatty acids during colonization of the host. On the basis of our results, it is tempting to speculate that host LDLs may support the growth of fatty acid auxotrophs of *S. aureus* during infection, providing a mechanism for the bypass of FASII inhibition *in vivo*. Consistent with this, spontaneous triclosan-resistant fatty acid auxotrophs have previously been isolated from clinical samples (24). Previous studies focused on the interactions between *S. aureus* and lipoprotein particles showed that LDLs defended against *S. aureus* infection by sequestering autoinducing peptide and phenol-soluble modulins (40–43, 70). Our studies expand on these findings by demonstrating that *S. aureus* is also capable of utilizing LDLs as a source of fatty acids.

Having established that *S. aureus* utilizes human LDLs as an exogenous fatty acid source, we turned our efforts to defining the molecular mechanism of this observation. Given that fatty acids of lipoprotein particles are esterified, we surveyed *S. aureus* lipase mutants for increased sensitivity to triclosan in the presence of LDLs. We observed that genetic inactivation of the lipase Geh reduced the ability of multiple strains of *S. aureus* to utilize LDLs as a source of exogenous fatty acids. Based on the decreased liberation of fatty acids from LDLs incubated with the *geh lip* mutant supernatant, we propose that the growth reduction of the *geh lip* mutant is due to an inability to liberate a sufficient quantity of fatty acids from the LDLs to overcome the effects of triclosan. Inactivation of *geh* does not ablate the ability of *S. aureus* to utilize LDL-derived fatty acids, indicating other potential mechanisms of fatty acid liberation from LDLs. Consistent with this, supernatants devoid of Geh and Lip still liberated fatty acids from human LDL, and a *geh lip* double mutant incorporated exogenous fatty acids into the cell membrane. Several hypothetical lipases encoded by the *S. aureus* genome remain uncharacterized and may also play a role in the liberation of fatty acids from lipoprotein particles (Table S5). Notably, purified bacterial lipases have been used in several atherosclerosis studies to liberate fatty acids from lipoprotein particles, and these bacterium-derived lipases have effects on lipoprotein particles similar to those of host lipases (71, 72). We explored the clinical relevance of our finding by evaluating the ability of lipase-positive and -negative clinical isolates of *S. aureus* to resist triclosan in the presence of lipoprotein particles. The loss of lipase activity in clinical isolates correlated with a reduced ability to use LDLs as an exogenous fatty acid source. The culmination of these studies supports the conclusion that *S. aureus* lipases liberate fatty acids from human LDLs.

FakA facilitates *S. aureus* incorporation of exogenous fatty acids into the membrane phospholipids (29). We reasoned that FakA is also necessary for LDLs to be used as an exogenous fatty acid source and found that the Δ*fakA* mutant is more sensitive to triclosan, when grown in the presence of exogenous fatty acids, than the WT. Moreover, genetic inactivation of *fakA* significantly impaired the ability of *S. aureus* to generate triclosan-resistant colonies on agar supplemented with free fatty acids. These results confirm that the molecular mechanism by which *S. aureus* utilizes exogenous fatty acids to resist triclosan treatment is FakA dependent. Additional support for this conclusion is based on the finding that *fakA* is necessary for LDL-dependent protection from triclosan. Together with the results obtained using *geh* and *geh lip* mutants, this finding implies that an active modification of human LDL-derived fatty acids is required for *S. aureus* triclosan resistance, reducing the possibility that protection occurs via a nonspecific triclosan-LDL interaction. A recent study using a murine systemic infection model with a *fakA* mutant found that this mutant is attenuated for colonization of the liver but not the kidneys (73). This finding is intriguing, considering our results demonstrating that FakA is necessary for the ability of *S. aureus* to utilize LDLs, as the

biogenesis of host lipoprotein particles occurs predominantly in the liver. In total, our results demonstrate that *S. aureus* lipases liberate fatty acids from human LDLs, which are then integrated into membrane PG in a *fakA*-dependent fashion.

In summary, we demonstrate that *S. aureus* scavenges fatty acids from human LDLs. While the ability of *S. aureus* to acquire fatty acids from its environment was well understood prior to this work, the fatty acid reservoirs present in the host exploited by *S. aureus* remained uncertain. LDLs and other lipoprotein particles are particularly relevant sources, as they are highly abundant in the blood and other tissues that *S. aureus* infects (74). The inhibition of FASII for treatment of *S. aureus* is the focus of much research, but debate continues regarding its clinical potential. This is because *S. aureus* and many other Gram-positive pathogens compensate for the loss of fatty acid synthesis by importing exogenous fatty acids. While our work is limited to *in vitro* studies, our results indicate that LDLs could support two possible routes to increase FASII inhibitor tolerance by *S. aureus*: (i) LDLs can supply fatty acids to WT cells via Geh and FakA in the presence of triclosan, which allows for the continued production of membrane phospholipids, and (ii) LDLs support the growth of fatty acid auxotrophs which, in a *fakA*-dependent manner, are insensitive to the effects of FASII inhibitors. Thus, this work also demonstrates that FakA is at the center of both possible mechanisms of FASII resistance in *S. aureus* and could possibly be a target for small-molecule inhibitors for use in conjunction with FASII inhibitors.

MATERIALS AND METHODS

Reagents. Most chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO), including triclosan (catalog number 72779), Tween 80 (catalog number P1454), palmitic acid (catalog number P5585), sodium oleate (catalog number O7501), sodium myristate (catalog number M8005), sodium chloride (catalog number S9625), and purified human LDL (catalog number L7914; lot number SLBT1781 at 6.73 mg of protein per ml, lot number SLBV0530 at 6.26 mg of protein per ml, lot number SLBW4532 at 6.37 mg of protein per ml, and lot number SLBV8079 at 6.3 mg of protein per ml). Ammonium sulfate was purchased from Thermo Fisher Scientific (catalog number BP212R; Waltham, MA). Egg yolk plasma separation was based on previously described protocols (75, 76). Chicken egg yolks were separated from the egg whites and rinsed twice using sterile phosphate-buffered saline (PBS). The egg yolk was transferred to filter paper, and the vitelline membrane was punctured using a sterile pipette tip. The contents of the vitelline membrane were drained into a sterile conical tube. The egg yolk was then mixed vigorously with an equal volume of sterile PBS. Centrifugation of diluted egg yolk was executed at 4°C for 1 h at 15,344 × *g* in a F14-6x250 rotor from Thermo Fisher Scientific (Waltham, MA). The plasma supernatant was removed, placed in a sterile test tube, and stored at 4°C. Egg yolk LDL isolation was performed as described by Moussa et al. (77). Briefly, the egg yolk was diluted in 2 volumes of 0.17 M NaCl and stirred for 1 h at 4°C. The diluted egg yolk was then centrifuged at 10°C for 45 min at 10,000 × *g*. The supernatant was then removed, placed in a sterile test tube, and mixed with 40% ammonium sulfate for 1 h at 4°C. The pH of the supernatant was adjusted to 8.7. The supernatant was then centrifuged at 4°C for 45 min at 10,000 × *g*. The resulting supernatant was dialyzed overnight in ultrapure water to remove the ammonium sulfate. Dialyzed supernatant was then centrifuged at 4°C for 45 min at 10,000 × *g*, and an LDL-rich floating residue was removed into a sterile tube and stored at 4°C.

Bacterial strains and growth conditions. The strains used in this study are described in Table 2. AH1263 is derived from a methicillin-resistant human clinical isolate of *S. aureus*, community-acquired methicillin-resistant *S. aureus* (CA-MRSA) USA300 LAC (78). AH1263 Δ*fakA* is an in-frame deletion mutant previously described by Bose et al. (62). Newman is a laboratory-derived methicillin-sensitive human isolate of *S. aureus* (79). The prophage-repaired variant of Newman, TB4, has been described by Bae et al. (60). The *aur* (NE163), *sspA* (NE1506), *geh* (NE1775), and *lip* (NE338) transposon mutants were obtained from the Nebraska Transposon Mutant Library (80). The backcrossed strains used in the assays described here were produced via phage-mediated transduction (80). For the AH1263 *geh lip* double mutant, the erythromycin resistance cassette was replaced with a tetracycline resistance cassette using a previously described allelic replacement strategy (81). The tetracycline-resistant *geh* allele was transduced into AH1263 *lip* harboring an erythromycin resistance allele. PCR was used to confirm that the transposon insertion disrupted the correct genes. Cells were grown in tryptic soy broth (catalog number 211822; BD) or tryptone broth at 37°C. Tryptone broth consisted of 1% tryptone (catalog number 211705; BD), 0.8% NaCl, and ultrapure water; for agar, 1.5% agarose (catalog number 214010; BD) was added to the broth. Mannitol salt agar (catalog number 211407; BD) was prepared following the manufacturer's directions. Baird-Parker agar (catalog number R452342; Remel) was prepared by following the manufacturer's directions.

Growth curves for all strains, except for the fatty acid auxotrophs (see below), were performed by diluting overnight cultures 1:100 into fresh broth and growing the diluted cells to an optical density (OD) at 600 nm (OD_{600}) of 0.5. Cells were then added to 96-well round-bottom plates at a final OD_{600} of 0.01. Growth was monitored as the optical density at 600 nm using an H1 BioTek plate reader set at 37°C with continuous, linear shaking.

TABLE 2 *S. aureus* strains used in this study

Strain[a]	Relevant characteristic(s)	Reference or source
MRSA USA300 strains		
Wild type	AH1263	78
fakA mutant	Δ*fakA*	62
aur mutant	*aur*::*erm*	This study
sspA mutant	*sspA*::*erm*	This study
geh mutant	*geh*::*erm*	This study
lip mutant	*lip*::*erm*	This study
geh lip mutant	*geh*::*tet lip*::*erm*	This study
AH1263 triclosan-resistant strains		
Trial 1, isolate A (1A)	*accB* fatty acid auxotroph	This study
Trial 1, isolate B (1B)	Fatty acid auxotroph	This study
Trial 1, isolate C (1C)	*accC* fatty acid auxotroph	This study
Trial 2, isolate A (2A)	*accA* fatty acid auxotroph	This study
Trial 2, isolate B (2B)	Fatty acid auxotroph	This study
Trial 2, isolate C (2C)	*accD* fatty acid auxotroph	This study
Trial 3, isolate A (3A)	Fatty acid auxotroph	This study
Trial 3, isolate B (3B)	Fatty acid auxotroph	This study
Trial 3, isolate C (3C)	*fadD accC* fatty acid auxotroph	This study
MSSA NM strains		
Wild type	Lipase negative	79
TB4	Prophage repaired, lipase positive	60
TB4 *geh* mutant	*geh*::*erm*	This study
Cystic fibrosis isolates		
CF:716	Lipase positive	This study
CF:720	Lipase negative	This study
CF:769	Lipase negative	This study
CF:781	Lipase negative	This study
CF:789	Lipase positive	This study
CF:796	Lipase positive	This study

[a]MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*.

Genomic DNA isolation and gene sequencing. Fatty acid auxotrophs were grown overnight in TSB supplemented with EYP. Cells were pelleted, resuspended in buffer, and incubated with lysostaphin from ABMI (Lawrence, NY) to remove the cell wall. Genomic DNA was isolated using a Promega Wizard genomic DNA purification kit (Madison, WI) following the manufacturer's directions. Amplification was performed using New England BioLabs Phusion HF DNA polymerase (Ipswich, MA) following the manufacturer's directions. Primers for *fabD* and *accA*, *accB*, *accC*, and *accD* were previously described by Morvan et al. (20). The following primers were added to our study to increase sequencing coverage: AccA-R (5′-CAGAATTTTTATTAGAGCATGGAC-3′) and AccD-F (5′-CTTCTTGTAAATCCACATCATTT-3′). Sanger sequencing was performed by the Michigan State University Research Technology Support Facility (East Lansing, MI).

Isolation of *S. aureus* from cystic fibrosis patient sputum. The collection of patient sputum samples was reviewed and approved by the Institutional Review Board of the Michigan State University Human Research Protection Program and performed according to NIH guidelines and U.S. federal law by Martha Mulks in the Department of Microbiology and Molecular Genetics at Michigan State University. Sputum samples are collected as part of the routine care of cystic fibrosis patients. The sputum samples were given a deidentification study number, mixed with an equal volume of 4% tryptone–40% glycerol freezing medium, and then archived at −80°C. Frozen sputum samples were streaked onto an *S. aureus* selective medium, mannitol salt agar. Isolated colonies of suspected *S. aureus* isolates were picked and streaked for a second time to mannitol salt agar and to the *S. aureus* selective medium Baird-Parker. Isolated colonies were lastly confirmed through positive Gram staining. These samples were characterized for lipase activity by spotting 5 μl of an overnight culture grown in TSB onto a TSA plate with or without 1% EYP and mannitol salt agar. The plates were incubated overnight at 37°C. On the following day, the colonies were inspected for the formation of a zone of clearing around the colony and compared to USA300 and Newman as controls.

Kirby-Bauer disk diffusion assay. Overnight cultures were normalized to an OD_{600} of 0.5, and a sterile cotton applicator was used to plate a lawn of cells on agar plates consisting of 20 ml of TSA or 1% tryptone agar with or without fatty acid supplementation. A sterile 6-mm filter disk was impregnated with 10 μl of 1.7 mM triclosan and allowed to dry. The sterile disk was then placed on top of the agar plate containing the cells. The plates were incubated for ~14 h at 37°C. The diameter of the zone of inhibition (in millimeters) was determined by measurement of the distance between the edges of the solid bacterial lawn using a digital caliper. The chemical fatty acid supplement consisted of 170 μM (each) palmitic acid, sodium oleate, and sodium myristate, as previously described by Morvan et al. (20). Additionally, 1% EYP was also used as a source of fatty acids.

IC_{50} assay. Overnight cultures of WT *S. aureus* or the *S. aureus fakA* mutant were diluted 1:100 into 96-well flat-bottom plates containing fresh TSB supplemented with 0.1% Tween 80 or 0.1% chicken LDLs or not supplemented and increasing concentrations of triclosan. Cultures were grown statically for ~16 h at 37°C. Cells were suspended by pipetting, and the optical density at 600 nm was measured. IC_{50}s were calculated with the aid of the computer program Prism (version 6.07; GraphPad Software, La Jolla, CA).

Generation and maintenance of triclosan-resistant mutants. Fatty acid-based triclosan-resistant mutants were generated by plating overnight cultures of WT strain AH1263 as a lawn on TSA with a fatty acid supplement (20). A 6-mm filter disk steeped with 10 μl of 1.7 mM triclosan was placed on top of the agar. The plates were incubated for 2 days at 37°C, and resistant colonies were counted each day. On day 2, colonies that grew in the zone of clearing were patched onto TSA or TSA containing the fatty acid supplement and incubated overnight at 37°C. To confirm that the triclosan-resistant mutants were fatty acid auxotrophs, the cells were patched onto TSA supplemented with fatty acids or not supplemented. Cells that grew on fatty acid-supplemented TSA were selected for further characterization. Fatty acid auxotrophs were maintained by streaking onto agar plates containing the fatty acid supplement or in broth cultures supplemented with 0.1% EYP. For growth assays, fatty acid auxotrophs were washed once with equal volumes of fresh TSB. Cells were then pelleted and resuspended in an equal volume of fresh TSB before being subcultured at 1:100 into a 96-well round-bottom plate containing TSB or TSB supplemented with LDLs.

Lipid extraction for mass spectrometry analysis of membrane phospholipid fatty acids of cells and culture supernatants. The culturing of cells for mass spectrometry analysis of exogenous fatty acid incorporation into the plasma membrane *of S. aureus* was based on a previously described protocol (22). Overnight cultures of AH1263 and AH1263 *geh lip* in tryptone broth were subcultured at 1:100 into 10 ml of fresh tryptone broth. The cultures were grown to mid-log phase, pelleted, and resuspended into 300 μl of fresh tryptone broth with or without 5% human LDLs. Each treatment condition was performed in triplicate. The cultures were grown for an additional 4 h before the cells were pelleted and washed twice in 2 volumes of sterile PBS. Mass spectrometry analysis of cultures performed in TSB was completed as described above, except that all culturing was performed with TSB.

To measure the lipase activity of the culture supernatants on human LDLs, 5-ml cultures of AH1263 and AH1263 *geh lip* were grown overnight in tryptone broth. These cultures were then pelleted twice. Each time, the top half of the supernatant was then carefully removed. Supernatants were then incubated briefly with 15 μg/ml chloramphenicol. Next, 300 μl of chemically sterilized supernatants was transferred into fresh sterile tubes and 5% human LDLs were added to each tube. The tubes were then incubated at 37°C for 2 h. Each treatment condition was performed in triplicate.

Monophasic lipid extraction in methanol-chloroform-water (2:1:0.74) was performed as previously described (82) using 15 to 20 mg (wet weight) of freshly grown *S. aureus*. Prior to lipid extraction, each sample was spiked with 10 nmol of synthetic phosphatidylserine (PS; 14:0/14:0) obtained from Avanti Polar Lipids (Alabaster, AL) for relative lipid quantitation. Dried lipid extracts were washed three times with 10 mM ammonium bicarbonate, dried under vacuum, and resuspended in methanol containing 0.01% butylated hydroxytoluene. For each analysis, 3 μl of lipid extract was transferred to an Eppendorf Twin-Tec 96-well plate (Sigma-Aldrich, St. Louis, MO) and evaporated under nitrogen. The dried lipid film was then resuspended in 30 μl isopropanol-methanol-chloroform (4:2:1, vol/vol/vol) containing 20 mM ammonium formate and sealed with Teflon ultrathin sealing tape (Analytical Sales and Services, Pompton Plains, NJ).

High-resolution/accurate mass spectrometry and tandem mass spectrometry. Untargeted lipidomic analysis utilized direct-infusion high-resolution/accurate mass spectrometry and tandem mass spectrometry. Samples were directly infused into the mass spectrometer by nanoelectrospray ionization (nESI) using a Triversa Nanomate nESI source (Advion, Ithaca, NY) with a spray voltage of 1.4 kV and a gas pressure of 0.3 lb/in^2. The mass spectrometry platform was a Thermo Scientific LTQ-Orbitrap Velos system (San Jose, CA). High-resolution MS and MS/MS spectra were acquired in the negative ion mode using an Fourier transform analyzer operating at 100,000 mass resolving power. Ion mapping higher-energy collision induced dissociation (HCD-MS/MS) product ion spectra were acquired to confirm lipid head groups and the acyl chain compositions.

Peak and lipid identification and quantification. Lipids were identified using the Lipid Mass Spectrum Analysis (LIMSA; version 1.0) software linear fit algorithm, in conjunction with an in-house database of hypothetical *S. aureus* and human-derived lipid compounds, for automated peak finding and correction of ^{13}C isotope effects. Values were reported only for molecular species that exhibited a full-scan MS signal-to-noise ratio of at least 3 in all biological replicates of each control or treatment group. The *S. aureus* lipid classes and molecular species used for database searches were derived from the published literature (54, 83). Relative quantification of abundance between samples was performed by normalization of target lipid ion peak areas to the PS (14:0/14:0) internal standard as previously described (84). For this untargeted analysis, no attempt was made to correct for differences in lipid species ionization due to head group polarity or the length or degree of unsaturation of the esterified fatty acids. Therefore, lipid abundance values are reported only as ion abundances after normalization against the internal standard rather than as absolute values.

Statistical analyses. Statistical significance was determined using Student's *t* test or a two-way analysis of variance (ANOVA) with a Tukey *post hoc* test for multiple comparisons and was performed with Prism software (version 6.07; GraphPad Software, La Jolla, CA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00728-17.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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